Inhibition of amylase secretion from differentiated AR4-2J pancreatic acinar cells by an actin cytoskeleton controlled protein tyrosine phosphatase activity

Peter Feick[a], Sven Gilhaus[a], Robert Blum[a], Fred Hofmann[b], Ingo Just[b], Irene Schulz[a,*]

[a] *Institut für Physiologie II, Universität des Saarlandes, D-66421 Homburg/Saar, Germany*
[b] *Institut für Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Hermann-Herder-Strasse 5, D-79104 Freiburg, Germany*

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Abstract Disruption of the actin cytoskeleton in AR4-2J pancreatic acinar cells led to an increase in cytosolic protein tyrosine phosphatase activity, abolished bombesin-induced tyrosine phosphorylation and reduced bombesin-induced amylase secretion by about 45%. Furthermore, both tyrosine phosphorylation and amylase secretion induced by phorbol ester-induced activation of protein kinase C were abolished. An increase in the cytosolic free Ca^{2+} concentration by the Ca^{2+} ionophore A23187 had no effect on tyrosine phosphorylation but induced amylase release. Only when added together with phorbol ester, the same level of amylase secretion as with bombesin was reached. This amylase secretion was inhibited by about 40% by actin cytoskeleton disruption similar to that induced by bombesin. We conclude that actin cytoskeleton-controlled protein tyrosine phosphatase activity downstream of protein kinase C activity regulates tyrosine phosphorylation which in part is involved in bombesin-stimulated amylase secretion.

Key words: Actin cytoskeleton; Protein tyrosine phosphatase; Amylase secretion; Tyrosine phosphorylation

1. Introduction

In different cell types, cellular responses to hormones and/or growth factors are mediated by the activation of tyrosine kinases [1]. Phosphorylation of tyrosine residues in specific proteins is due to the activity not only of tyrosine kinases, but also of protein tyrosine phosphatases (PTPases). Specific PTPase inhibitors, such as pervanadate [2], are useful tools in the study of hormone- and growth factor-induced protein tyrosine phosphorylation. Recently, we have shown that incubation of differentiated rat pancreatic AR4-2J cells with pervanadate, similar to bombesin, leads to amylase secretion and to tyrosine phosphorylation of $p125^{FAK}$ and paxillin [3]. Our data suggested that protein tyrosine phosphorylation downstream of protein kinase C (PKC) activation is involved in stimulation of enzyme secretion from these cells [3].

Previously, it has been reported that in Swiss 3T3 cells, an increased tyrosine phosphorylation of $p125^{FAK}$ and paxillin is accompanied by a profound reorganization of the actin cytoskeleton and by the assembly of focal adhesion plaques, distinct sites in the plasma membrane where both $p125^{FAK}$ and paxillin are localized [4,5]. Studies using cytochalasin D, which selectively disrupts the actin cytoskeleton network, demonstrated that bombesin-induced tyrosine phosphorylation of $p125^{FAK}$ and paxillin depends on the integrity of the actin cytoskeleton [4,6]. Work during the past 5 years has shown that small GTP-binding proteins of the Ras superfamily, the Rho proteins Rho, Rac and Cdc42, are involved in the control of the actin cytoskeleton organization (reviewed in [7]). Small GTP-binding proteins and actin are selectively modified and thereby inactivated by clostridial cytotoxins [8–10]. *Clostridium botulinum* exoenzyme C3 is a transferase which inactivates Rho (RhoA, RhoB, RhoC) by ADP-ribosylation at Asn^{41} [11]. Both toxin A and toxin B from *Clostridium difficile* monoglucosylate Rho, Rac and Cdc42 by transfer of glucose to threonine in the effector domain (Thr^{37} of Rho) [9,10]. In contrast to these toxins, the C2 toxin of *C. botulinum* directly ADP-ribosylates actin at Arg^{177} [8]. Incubation of cells with each of these toxins leads to the depolymerization of actin filaments and destruction of the actin cytoskeleton [8–10].

It was the purpose of this study to investigate if the actin cytoskeleton organization plays a role in the signal cascade of stimulus-secretion coupling in rat pancreatic AR4-2J acinar cells. Our data show that destruction of the actin cytoskeleton leads to an increase in the cytosolic PTPase activity. Consequently, bombesin-induced tyrosine phosphorylation of both $p125^{FAK}$ and paxillin is decreased. Destruction of the actin cytoskeleton also leads to a reduction of about 45% in bombesin-stimulated amylase release and abolishes amylase release stimulated by the PKC activator phorbol 12-myristate 13-acetate (PMA) after 30 min of incubation, whereas the Ca^{2+}-induced amylase secretion is not affected. We assume that regulation of the organization of the actin cytoskeleton, which in turn controls the PTPase activity, plays an important role in protein secretion from pancreatic AR4-2J cells.

2. Materials and methods

2.1. Materials

C. difficile toxin B was prepared as described [12]. Genistein, cytochalasin D, calcium ionophore A23187 and PMA were obtained from Calbiochem (Bad Soden, Germany) and were prepared in dimethyl sulfoxide as stock solutions. Anti-phosphotyrosine antibody (clone PY20) was purchased from Santa Cruz (Heidelberg, Germany) and anti-$p125^{FAK}$ antibody (clone 2A7) from Biomol (Hamburg, Germany). Anti-paxillin antibody (clone 349) and protein G PLUS/protein A-agarose were obtained from Dianova (Hamburg). Peroxide of vanadate (pervanadate) was prepared by mixing vanadate (Sigma, Deisenhofen, Germany) with H_2O_2 (Merck, Darmstadt, Germany) for 15 min at room temperature, followed by the addition of catalase

*Corresponding author. Fax: (49) (6841) 166655.
E-mail: schulz@med-ph.uni-sb.de

Abbreviations: PTPase, protein tyrosine phosphatase; PKC, protein kinase C; FAK, focal adhesion kinase; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium

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(Sigma) to remove residual H_2O_2 as described by Fantus et al. [13]. Fetal calf serum was obtained from PAA Laboratories (Cölbe, Germany) and penicillin/streptomycin from Gibco (Eggenstein, Germany). Leupeptin was purchased from Serva (Heidelberg) and trypsin inhibitor (hen egg white) from Boehringer (Mannheim). Dulbecco's modified Eagle's medium (DMEM) was from Sigma. All the other reagents were of analytical grade and commercially available.

2.2. Cell culture and treatment with toxins

AR4-2J cells were obtained from the American Type Culture Collection (ATCC number CRL 1492) (Rockville, MD, USA). The cells were seeded at 750 000 cells per 35 mm Petri dish and were routinely grown for 72 h in DMEM supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation of the cells was induced by addition of 100 nM dexamethasone. After 48 h, the medium was changed and the cells were incubated with toxins (*C. difficile* toxin B or cytochalasin D) or without toxins for the indicated times. After toxin treatment, cells were washed with the appropriate buffer and used for the assays.

2.3. Amylase release

For measurement of the amylase release, toxin-treated and untreated AR4-2J cells were washed three times with KRH buffer (which contained in mM: 130 NaCl, 5 KCl, 2 $MgCl_2$, 1.2 KH_2PO_4, 1 $CaCl_2$, 20 HEPES, 10 glucose, 0.1 mg/ml trypsin inhibitor, pH 7.4) and pre-incubated for 10 min at 37°C. The cells were stimulated by addition of agonists and at indicated times, aliquots of the supernatants were removed for the determination of amylase released by the cells. For measurement of the total amount of amylase, the cells were scraped off and lysed in a buffer containing (in mM) 5 HEPES, pH 7.0, 280 mannitol, 10 KCl, 1 $MgCl_2$, 1 benzamidine, 0.001 leupeptin, 0.2 PMSF, 20 μg/ml trypsin inhibitor and 0.1% (v/v) Triton X-100. The amylase activity was determined using the Phadebas Amylase Test (Pharmacia, Freiburg, Germany) and was expressed as percentage of the total cellular content of amylase present at the beginning of the incubation.

2.4. Tyrosine phosphorylation and immunoprecipitation

For the determination of tyrosine phosphorylation, toxin-treated and untreated AR4-2J cells were washed three times with KRH buffer and pre-incubated with or without tyrosine kinase inhibitor for 10 min at 37°C. Bombesin or pervanadate were added for the indicated times followed by washing the cells with ice-cold PBS containing 0.5 mM sodium orthovanadate. Cells were then scraped off and lysed in a lysis buffer containing (in mM) 10 Tris-HCl, pH 7.5, 50 NaCl, 5 EDTA, 0.5 sodium orthovanadate, 30 sodium pyrophosphate, 50 NaF, 1 PMSF, 0.5 μg/ml leupeptin and 0.5% (v/v) Triton X-100. After sonication of the cell lysates, three times concentrated electrophoresis sample buffer [14] was added and the samples were boiled for 5 min to denature proteins. For immunoprecipitation, 500 μl lysate containing 500 μg of total protein was incubated with 1.5–3 μg primary antibody for 60 min on ice. After this time, 30 μl of a 30% suspension of protein G-PLUS/protein A-agarose in PBS was added and the samples were incubated for an additional 60 min with agitation at 4°C. The agarose beads were centrifuged and washed three times with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% (v/v) Triton X-100. Then, the beads were resuspended in electrophoresis sample buffer [14] and boiled for 5 min to release precipitated proteins from the beads.

2.5. In vitro PTPase assay

AR4-2J cells were incubated with toxin B (10 ng/ml) or 5 μM cytochalasin D for 20 h. After washing with ice-cold PBS, cells were harvested in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF and 0.5 μg/ml leupeptin and sonicated twice for 5 s on ice. Then, cell homogenates were centrifuged for 5 min at 4°C and $350\times g$. The supernatant was further centrifuged for 15 min at 4°C and $30\,000\times g$ and the resulting supernatant was used as 'cytosol' for determination of the PTPase activity. As substrate, paxillin was tyrosine-phosphorylated by incubation of cells with 100 μM pervanadate for 15 min and immunoprecipitated with 1.5 μg anti-paxillin antibody from 400 μg total cell protein as described above. This immunoprecipitated paxillin was incubated for up to 45 min with 'cytosol' (0–1.5 mg/ml protein) obtained from untreated or toxin-treated cells in the absence or presence of pervanadate as indicated. The reaction was terminated by addition of half the volume of three times concentrated electrophoresis sample buffer and by boiling for 5 min to denature the proteins.

2.6. Electrophoresis and immunoblotting

Proteins were electrophoretically separated in SDS-polyacrylamide gels [14]. Electrotransfer to nitrocellulose membranes for 60 min at 0.8 mA/cm^2 was done in a buffer which contained 48 mM Tris, 39 mM glycine, pH 8.6, 0.0375% (w/v) SDS and 20% (v/v) methanol. Membranes were blocked using 1% (w/v) bovine serum albumin in Tris-buffered saline (10 mM Tris-HCl, pH 8.0 and 150 mM NaCl) for 60 min and incubated for 60 min with primary antibodies diluted in Tris-buffered saline plus 0.2% (v/v) Tween 20. Bound antibodies were visualized with secondary antibodies conjugated to horseradish peroxidase using an enhanced chemiluminescence detection kit (Amersham, Braunschweig, Germany) and Reflection autoradiography film (NEN Life Science Products). The density of bands on the film was measured using a scanning densitometer (Fröbel Labortechnik, Lindau, Germany).

2.7. Protein determination

Protein was assayed as described by Bradford [15] using bovine serum albumin as standard.

3. Results and discussion

3.1. The effect of toxin B on bombesin- and pervanadate-induced protein tyrosine phosphorylation

Our recent studies support the view that protein tyrosine phosphorylation is involved in secretagogue-induced amylase secretion from AR4-2J cells [3]. Rozengurt and coworkers have shown that the integrity of the actin cytoskeleton is essential for hormone-induced protein tyrosine phosphorylation in Swiss 3T3 cells [4,6]. We therefore examined if the actin cytoskeleton organization affects the protein secretion in AR4-2J cells by the use of *C. difficile* toxin B and cytochalasin D. Toxin B has been identified as monoglucosyltransferase [9] and has been shown to inactivate Rho proteins [16]. After treatment of AR4-2J cells with toxin B (10 ng/ml) for 20 h, all target proteins known for toxin B-induced inactivation were found to be nearly completely glucosylated in intact AR4-2J cells (results not shown). The inactivation of Rho proteins by toxin B resulted in a rounding up of the cells indicating a change in the organization of their actin cytoskeleton (results not shown). Similarly, treatment of AR4-2J cells with 5 μM cytochalasin D, which directly inhibits actin polymerization, changed the organization of the actin cytoskeleton of AR4-2J cells (results not shown).

Using a monoclonal antibody to phosphotyrosine, we examined by immunoblot analysis if the actin filament destroying toxins affects the hormonal-induced protein tyrosine phosphorylation in AR4-2J cells. As shown in Fig. 1A, left panel, AR4-2J cells stimulated with bombesin for 15 min showed a significant increase in tyrosine phosphorylation of proteins with an apparent molecular mass of 120–140 kDa and of about 70 kDa. In the presence of toxin B, bombesin-induced tyrosine phosphorylation of these proteins was completely inhibited, similar as with the tyrosine kinase inhibitor genistein. In the presence of cytochalasin D, also a complete inhibition of bombesin-induced tyrosine phosphorylation was observed. In addition, there was a strong increase in tyrosine phosphorylation of proteins in the range of 145 kDa and a small increase in tyrosine phosphorylation of proteins of 85 and 56 kDa. The identity of these protein bands cannot be assigned at present.

Reduction in the bombesin-induced protein tyrosine phos-

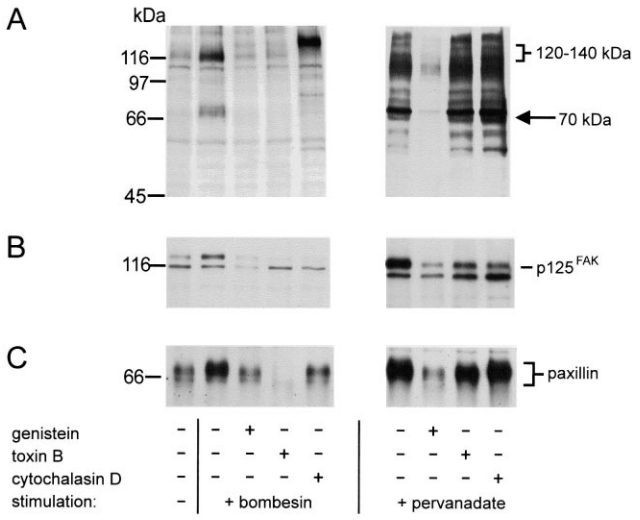

Fig. 1. The effect of genistein, *C. difficile* toxin B and cytochalasin D on bombesin- or pervanadate-induced tyrosine phosphorylation of $p125^{FAK}$ and paxillin. Differentiated AR4-2J cells were incubated in DMEM in the absence or presence of toxin B (10 ng/ml) for 21 h or with 5 μM cytochalasin D for 2 h. Cells were then washed with KRH buffer and pre-incubated in the absence or presence of 100 μM genistein for 10 min at 37°C. Then, cells were treated with 10 nM bombesin or with 100 μM pervanadate for 15 min at 37°C. After lysis of the cells, immunoprecipitations of $p125^{FAK}$ and paxillin were performed. Total cell lysates (A) and immunoprecipitates of $p125^{FAK}$ (B) and paxillin (C) were subjected to SDS-PAGE and immunoblotting was performed using anti-phosphotyrosine antibodies as described in Section 2. The experiment shown is representative for three (immunoprecipitation) and four (total cell lysates) separate experiments, respectively.

phorylation by toxin B or cytochalasin D could be either due to the inhibition of tyrosine kinase activity, similar as with genistein, and/or due to an increase in PTPase activity which overrides the bombesin-stimulated tyrosine kinase activity. To check both possibilities, AR4-2J cells pre-treated without or with either genistein, toxin B or cytochalasin D were incubated with 100 μM pervanadate for 15 min to inhibit PTPases. As reported previously [3] and shown in Fig. 1B, right panel, pervanadate induced tyrosine phosphorylation of multiple proteins including 120–140 and 70 kDa proteins. Whereas genistein reduced the total protein tyrosine phosphorylation by about 95%, treatment of the cells with toxin B or cytochalasin D had no effect on the pervanadate-induced protein tyrosine phosphorylation.

The focal adhesion-associated proteins $p125^{FAK}$ and paxillin are the most prominent tyrosine-phosphorylated proteins in bombesin-treated AR4-2J cells [3]. As shown in Fig. 1B, left panel, bombesin-induced tyrosine phosphorylation of $p125^{FAK}$ was about 5-fold higher than the control and was completely inhibited by treatment of cells with each of the toxins and inhibitors tested. Bombesin-induced tyrosine phosphorylation of paxillin was about 3-fold higher than the control. It was reduced to basal levels by treatment of the cells with genistein or cytochalasin D and completely inhibited by toxin B treatment (Fig. 1C, left panel). The tyrosine phosphorylation of $p125^{FAK}$ induced by pervanadate was about 30-fold of the control and was reduced by about 95% by genistein and surprisingly also inhibited by about 40 and 50% by toxin B or cytochalasin D, respectively (Fig. 1B, right panel). The pervanadate-induced tyrosine phosphorylation of paxillin was about 12-fold of the control and was reduced by about 90% by genistein, but was significantly reduced neither by toxin B nor by cytochalasin D (Fig. 1C, right panel).

Since these toxins lead to depolymerization of the actin cytoskeleton, we conclude that an intact actin cytoskeleton inhibits the PTPase activity. In order to test if the destruction of the actin cytoskeleton increases the PTPase activity, cytosol from control and toxin-treated cells was incubated with highly tyrosine-phosphorylated paxillin prepared by immunoprecipitation after incubation of the cells with pervanadate. The phosphotyrosine content of paxillin was analyzed after SDS-PAGE and immunoblotting using the monoclonal anti-phosphotyrosine antibody. As shown in Fig. 2A, in the presence of cytosol prepared from toxin B- or cytochalasin D-treated cells, the tyrosine phosphorylation of paxillin was time-dependently reduced, whereas cytosol from untreated cells caused only a small reduction in tyrosine phosphorylation of paxillin. Tyrosine phosphorylation of paxillin was not reduced by incubation with cytosol from untreated or toxin-treated cells when cytosol was pre-incubated with 100 μM of the PTPase inhibitor pervanadate for 10 min, indicating that the reduction in tyrosine phosphorylation of paxillin is

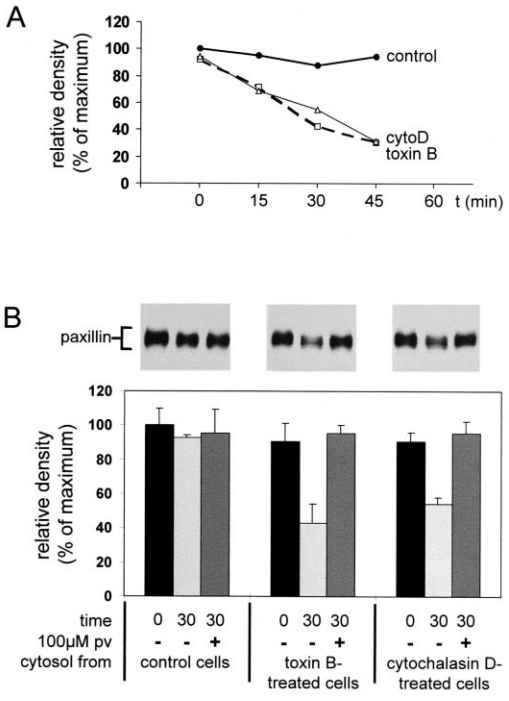

Fig. 2. The effect of *C. difficile* toxin B and cytochalasin D on PTPase activity in the cytosol of AR4-2J cells. Tyrosine-phosphorylated paxillin which was prepared as described in Section 2 was incubated with cytosol (1.33 mg/ml protein) from untreated (control) or toxin-treated cells (10 ng/ml toxin B, 5 μM cytochalasin D for 20 h) for the indicated times (A) in the absence or presence of 100 μM pervanadate (pv) for 30 min at 25°C (B). After termination of the reaction, samples were subjected to SDS-PAGE and the level of tyrosine phosphorylation of paxillin was determined following immunoblotting using anti-phosphotyrosine antibodies as described in Section 2. The immunoblot shown in the upper panel is a representative of three separate experiments. To quantify the level of tyrosine phosphorylation, the density of bands on the film was determined using a scanning densitometer. The values (mean ± S.D.) from three independent experiments are shown in the lower panel and are expressed as percentage of maximal tyrosine phosphorylation of paxillin.

due to PTPase activity present in the cytosol (Fig. 2B). Probing the same samples with an anti-paxillin antibody detected equal amounts of paxillin, indicating that immunoprecipitated paxillin was not degraded by incubation with cytosol from toxin B- or cytochalasin D-treated cells (results not shown). These results indicate that an intact actin cytoskeleton suppresses or negatively regulates PTPase activity and that disruption of the actin cytoskeleton ceases PTPase inhibition. Our data are in agreement with a report which suggests that assembly of the actin cytoskeleton plays a role in inhibition of PTPases involved in $p125^{FAK}$ tyrosine phosphorylation in human endothelial and Chinese hamster ovary cells [17].

In addition to a reduction in the pervanadate-induced $p125^{FAK}$ tyrosine phosphorylation by cytosol from toxin B- or cytochalasin D-treated cells (Fig. 1B, left panel), a small reduction in tyrosine phosphorylation could be observed even at inhibited cytosolic tyrosine phosphatase in the presence of pervanadate (Fig. 1B, right). This indicates that at least one specific tyrosine kinase is partially inhibited by an actin cytoskeleton disruption whereas the overall tyrosine kinase activity is not affected (Fig. 1A, right panel). In order to further test if destruction of the actin cytoskeleton inhibits the tyrosine kinase activity, we incubated AR4-2J cells with toxin B for 20 h. To stimulate tyrosine kinase activities, untreated and toxin B-pre-treated cells received bombesin for 15 and 30 min. Cytosol was prepared from these cells and was added to phosphorylated and immunoprecipitated $p125^{FAK}$ or paxillin in the presence of 0.5 mM ATP, 5 mM $MgCl_2$ and with 100 μM pervanadate to inhibit PTPases. The phosphotyrosine content of $p125^{FAK}$ and paxillin was analyzed after SDS-PAGE and immunoblotting using the monoclonal anti-phosphotyrosine antibody. Tyrosine phosphorylation of $p125^{FAK}$ and paxillin was increased about 2-fold in the presence of cytosol from cells which were stimulated with bombesin for 15 min as compared to cytosol from unstimulated cells. However, cytosol prepared from toxin B-pre-treated and bombesin-stimulated cells increased tyrosine phosphorylation of $p125^{FAK}$ and paxillin only by about 1.3-fold (results not shown). This indicates that actin cytoskeleton disruption in addition to tyrosine phosphatase activation also inhibits to some extent the tyrosine kinase activity.

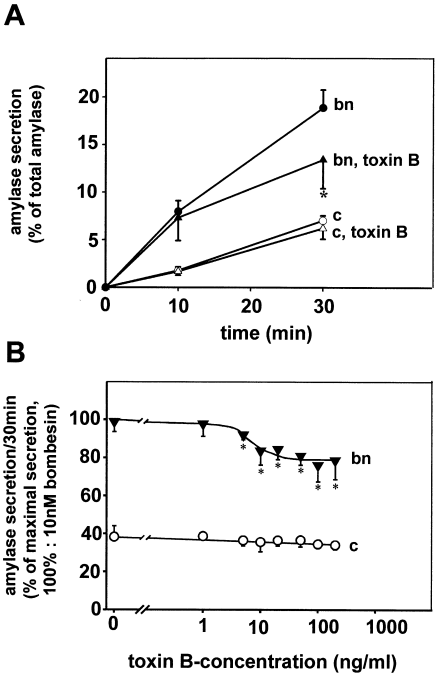

Fig. 3. The effect of *C. difficile* toxin B on unstimulated and bombesin-stimulated amylase release from AR4-2J cells. A: Differentiated AR4-2J cells were incubated in DMEM in the absence or presence of toxin B (10 ng/ml) for 21 h. Then, cells were washed and incubated with KRH buffer for up to 30 min as control without (c) or with 10 nM bombesin (bn). At indicated times, aliquots were removed from the medium and amylase secreted into the medium was quantified. The amylase release is expressed as percentage of the total amylase content of the cells present at the beginning of the incubation (mean ± S.D., $n = 5$). B: Differentiated AR4-2J cells were incubated in DMEM with indicated toxin concentrations for 21 h. Then, cells were washed and incubated in KRH buffer for 30 min at 37°C without (circles) and with 10 nM bombesin (triangles). Amylase secreted into the medium after 30 min of incubation was quantified and is expressed as percentage of the maximal amylase secretion induced by 10 nM bombesin (= 100%) (mean ± S.D., $n = 4$). The significance of the differences between values from untreated and toxin-treated cells was calculated using the *t*-test for paired values: $^*P < 0.01$.

3.2. The effect of toxin B on amylase secretion

In Fig. 3, the effect of toxin B on amylase secretion is illustrated. After 30 min of incubation, amylase secretion from differentiated AR4-2J cells was stimulated by a maximally effective concentration of 10 nM bombesin from 7.0 ± 0.5% of total amylase in control cells to 18.9 ± 1.9% (Fig. 3A). Pre-treatment of cells with toxin B (10 ng/ml) for 21 h showed no significant difference in the bombesin-stimulated secretion as compared to untreated cells within the initial 10 min. However, after 30 min of stimulation, the amylase secretion was reduced from 18.9 ± 1.9% to 13.4 ± 3.0% of total amylase ($P < 0.01$) in toxin B-treated cells. As shown in Fig. 3B, bombesin-stimulated secretion was not further inhibited at higher concentrations of up to 200 ng/ml toxin B, indicating that the small inhibition of bombesin-stimulated amylase secretion could not be due to insufficient application of toxins. Unstimulated amylase secretion was not affected by any of the tested toxin B concentrations. The partial inhibition of the protein secretion by toxin B is also not due to incomplete inactivation of Rho proteins, since all target proteins known for toxin B-induced inactivation were found to be nearly completely glucosylated in intact AR4-2J cells by a toxin B concentration of 10 ng/ml (results not shown).

Two phases of exocytosis are proposed to occur in pancreatic acinar cells: an initial phase, which is completed within 5–10 min of agonist stimulation, and a second phase, which is sustained for the duration of hormonal stimulation [18]. Whereas the initial rapid phase is mediated by production of inositol 1,4,5-trisphosphate followed by Ca^{2+} release from intracellular stores and Ca^{2+} influx into the cell, the sustained phase is associated with production of diacylglycerol and activation of PKC [19,20]. Artificial activation of these pathways by elevation of the cytosolic Ca^{2+} concentration in the presence of stimulators of PKC, such as the phorbol ester PMA, could mimic the effect of secretagogues on the enzyme secretion [20–22]. In order to test if the partial inhibition of amylase secretion by toxin B can be ascribed to inhibition of either phase, we examined the amylase release from toxin B pre-treated AR4-2J cells stimulated by an increase in the cytosolic free Ca^{2+} concentration and PKC activation. As

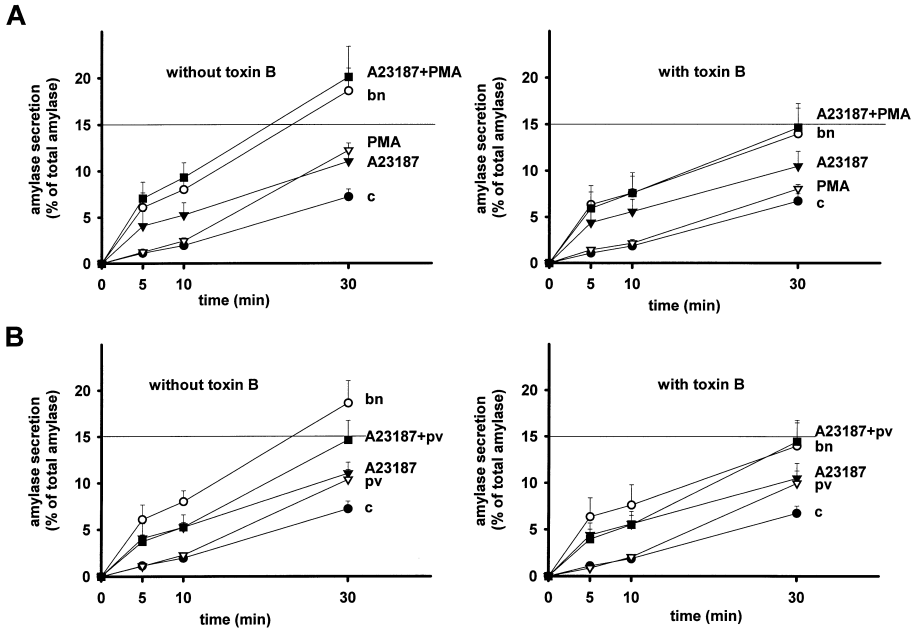

Fig. 4. The effect of *C. difficile* toxin B on PMA-, pervanadate- and calcium ionophore A23187-stimulated amylase secretion. Differentiated AR4-2J cells were incubated in DMEM in the absence or presence of toxin B (10 ng/ml) for 20 h. Then, cells were washed with KRH buffer and were left unstimulated (addition of a corresponding volume of vehicle, control (c)) or were stimulated by the addition of 10 nM bombesin (bn), 400 nM PMA, 100 μM pervanadate or 5 μM A23187 alone or in combinations as indicated. Amylase secretion over 30 min was measured as described in the legend to Fig. 1. For clarity, the effect of PMA on the amylase release is shown in A and the effect of pervanadate on amylase release is shown in B. Data shown in A and B are from the same series of experiments ($n = 4$).

shown in Fig. 4A, an increase in the cytosolic free Ca^{2+} concentration by the calcium ionophore A23187 increased the amylase secretion within the first 10 min and thereafter showed no higher secretion rate than the unstimulated control cells. However, A23187 enhanced the PMA-stimulated amylase release to levels comparable to those induced by bombesin, whereas PMA alone increased amylase release only after 10 min of incubation. Pre-treatment of cells with toxin B nearly completely abolished PMA-stimulated amylase release, whereas amylase secretion induced by A23187 alone was not inhibited. Similar to inhibition of bombesin, PMA plus A23187-induced amylase release was also inhibited by 40%. These data demonstrate that toxin B affects the PKC-stimulated but not the Ca^{2+}-stimulated pathway leading to amylase secretion. Our recent data suggest that in the cascade of events that leads to bombesin-induced protein secretion in AR4-2J cells, protein tyrosine phosphorylation occurs downstream of PKC [3]. Tyrosine phosphorylation of paxillin induced by PMA started after about 10 min of incubation and reached after 30 min the level of maximal tyrosine phosphorylation that was induced by bombesin (results not shown). The onset of paxillin tyrosine phosphorylation correlated with PMA-induced amylase release which began after 10 min (Fig. 4A) and was in the same range as the amylase release induced by bombesin after 60 min of incubation [3]. Protein tyrosine phosphorylation induced by bombesin (Fig. 1) or PMA (results not shown) is completely inhibited by treatment of the cells with toxin B due to an increased tyrosine phosphatase activity. Inhibition of PTPase activity by pervanadate resulted in a tyrosine phosphorylation which was inhibited by toxin B to some extent only due to partial inhibition of a tyrosine kinase (Fig. 1B, right). Inhibition of tyrosine dephosphorylation in the presence of pervanadate led to amylase secretion, which was nearly identical to that induced by PMA and was additive to that induced by A23187 (Fig. 4B) [3]. These data suggest that both protein tyrosine phosphorylation and the increase in the cytosolic free Ca^{2+} concentration act synergistically in stimulated amylase secretion. Treatment of cells with toxin B did not inhibit amylase secretion induced by pervanadate alone or in combination with A23187 (Fig. 4B). These observations suggest that partial inhibition (35–45%) of bombesin-stimulated amylase secretion by toxin B may be due to complete inhibition of protein tyrosine phosphorylation. The pathway stimulated by Ca^{2+} is not affected by actin cytoskeleton disruption. Therefore, about 55–65% of the bombesin-stimulated amylase release remains unaffected in the presence of toxin B. This result is opposite to that of Jena et al. [23] who showed that the introduction of a recombinant form of rat brain PTPase (rrbPTP-1) into permeabilized rat pancreatic acinar cells increased the Ca^{2+}-mediated secretion. Since the actin cytoskeleton-controlled PTPase has not yet been identified, the apparent discrepancy with the studies of Jena et al. [23] awaits further clarification.

Taken together, the results of this study demonstrate a striking correlation between actin cytoskeleton formation, protein tyrosine phosphorylation and secretion. We assume that activation of PTPase(s) by disassembly of the actin cytoskeleton leads to protein tyrosine dephosphorylation and consequent inhibition of protein secretion. Protein tyrosine phosphorylation occurs in a Ca^{2+}-independent manner downstream of PKC activation, whereas Ca^{2+} plays a role in the final step of exocytosis [3]. At present, neither the tyrosine kinase nor the PTPase, which could be involved in protein secretion, has been identified. If $p125^{FAK}$ and/or paxillin, which are mainly tyrosine-phosphorylated in the presence of bombesin, are involved in stimulus-secretion coupling in AR4-2J cells has to be investigated in future studies.

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